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DEK oncogene expression during normal hematopoiesis and in Acute Myeloid Leukemia (AML)

Logan, G. E., Mor-Vaknin, N., Braunschweig, T., Jost, E., Schmidt, P. V., Markovitz, D. M., Mills, K. I., Kappes, F., & Percy, M. J. (2015). DEK oncogene expression during normal hematopoiesis and in Acute Myeloid Leukemia (AML). *Blood Cells, Molecules and Diseases*, 54(1), 123-131.
<https://doi.org/10.1016/j.bcmd.2014.07.009>

Published in:
Blood Cells, Molecules and Diseases

Document Version:
Publisher's PDF, also known as Version of record

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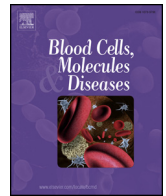
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Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmdDEK oncogene expression during normal hematopoiesis and in Acute Myeloid Leukemia (AML)[☆]Gemma E. Logan^a, Nirit Mor-Vaknin^b, Till Braunschweig^c, Edgar Jost^d, Pia Verena Schmidt^d, David M. Markovitz^b, Ken I. Mills^a, Ferdinand Kappes^{e,1}, Melanie J. Percy^{a,f,*}^a Centre for Cancer Research and Cell Biology (CCRCB), Queen's University Belfast, Belfast, United Kingdom^b Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA^c Institute of Pathology, Medical School, RWTH Aachen University, Aachen, Germany^d Clinic for Oncology, Hematology and Stem Cell Transplantation, Medical School, RWTH Aachen University, Aachen, Germany^e Institute of Biochemistry and Molecular Biology, Medical School, RWTH Aachen University, Aachen, Germany^f Haematology Department, Belfast City Hospital, Belfast Health and Social Care Trust, Belfast, United Kingdom

ARTICLE INFO

Article history:

Submitted 11 July 2014

Accepted 15 July 2014

Available online xxxx

(Communicated by M. Narla, DSc, 15 July 2014)

Keywords:

DEK

Acute Myeloid Leukemia

Translocation t(6;9)

Hematopoiesis

Leukemagenesis

ABSTRACT

DEK is important in regulating cellular processes including proliferation, differentiation and maintenance of stem cell phenotype. The translocation t(6;9) in Acute Myeloid Leukemia (AML), which fuses DEK with NUP214, confers a poor prognosis and a higher risk of relapse. The over-expression of DEK in AML has been reported, but different studies have shown diminished levels in pediatric and promyelocytic leukemias. This study has characterized DEK expression, *in silico*, using a large multi-center cohort of leukemic and normal control cases. Overall, DEK was under-expressed in AML compared to normal bone marrow (NBM). Studying specific subtypes of AML confirmed either no significant change or a significant reduction in DEK expression compared to NBM. Importantly, the similarity of DEK expression between AML and NBM was confirmed using immunohistochemistry analysis of tissue microarrays. In addition, stratification of AML patients based on median DEK expression levels indicated that DEK showed no effect on the overall survival of patients. DEK expression during normal hematopoiesis did reveal a relationship with specific cell types implicating a distinct function during myeloid differentiation. Whilst DEK may play a potential role in hematopoiesis, it remains to be established whether it is important for leukemagenesis, except when involved in the t(6;9) translocation.

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Introduction

Acute Myeloid Leukemia (AML) is primarily a hematological malignancy of the elderly with a median age of onset at 60 years and a poor prognosis with a five year survival rate of only 12% [1]. It is characterized by the accumulation of immature myeloblasts as a consequence of arrested myeloid differentiation and a subsequent deficiency in functional mature granulocytes and monocytes. AML often arises from chromosomal translocations resulting in specific leukemia-associated fusion proteins. These chimeric gene products exhibit distinctive functions that impede upon normal cellular proliferation

and/or differentiation and are utilized to classify AML into specific subtypes and risk groups of favorable, intermediate and adverse. The rare translocation t(6;9), present in 1–5% of AML cases [2], results in the production of the DEK-NUP214 (formerly CAN) fusion, which is associated with a particularly poor prognosis and a median age of 44 years at diagnosis. The DEK oncogene was originally identified from this leukemic translocation, where the 5' portion of the DEK gene located on chromosome 6p23 was fused to the 3' region of the NUP214 gene found on chromosome 9q34 resulting in the 165 kDa DEK-NUP214 fusion [3]. The leukemogenic potential of the DEK-NUP214 protein was undecided as it was unable to completely block differentiation of hematopoietic progenitors [4]. Subsequent data from Oancea *et al.* indicated DEK-NUP214 could promote leukemic transformation of a subset of long term repopulating hematopoietic stem cells [5], clearly pointing to an important contribution of DEK-NUP214 to leukemia. Data from two studies have revealed that the expression of DEK-NUP214 may increase the overall protein production by targeting translation [6], and may additionally accelerate proliferation through up-regulation of the mTOR pathway [7]. A recent international multicenter study has concluded that DEK-NUP214 represents a unique subtype of AML accompanied by increased risk of relapse,

[☆] All authors have no conflict of interest to declare.

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higher FMS-like tyrosine kinase 3 internal tandem duplication (FLT3 ITD) mutation frequency and a defined gene signature [8]. However, the precise molecular function of this fusion gene and its disease contribution remain mostly elusive.

Human DEK, 43 kDa in size, is an abundant and primarily chromatin-associated nuclear factor [9]. DEK exhibits a wide variety of molecular functions (e.g. regulation of gene expression, RNA biology, DNA repair, apoptosis, senescence, and chromatin structure), suggesting that it is critically involved in a myriad of cellular processes that relate to proliferation, differentiation, senescence and the maintenance of cell stemness [10]. Currently, it is believed that these functions are predominantly transmitted by the architectural functions of DEK within cellular DNA and chromatin [10]. DEK has two distinct DNA-binding domains (SAP-box and C-terminal DNA binding domain), which can induce intra- and intermolecular contacts that lead to the alteration of DNA and chromatin topology [11–13]. It is thought that changes to cellular DEK levels are most likely involved in regulating genomic stability and gene expression through concerted action of epigenetic mechanisms and chromatin architectural functions [10].

Although DEK predominately localizes in the nucleus under steady-state conditions, induced DNA damage, apoptosis and pro-inflammatory environments lead to its presence in the extracellular space, either through passive release in apoptosis by T-cells or active secretion from macrophages [14–16]. Extracellular DEK, in turn, gains novel functions, exhibiting chemo-attractant properties, resulting in the attraction of certain immune cells such as leukocytes of the immune system to the site of inflammation [15,16]. It has been shown recently by the addition of exogenous recombinant DEK that it can also mediate functions of hematopoietic stem cells (HSC) by suppressing proliferation of hematopoietic progenitor cells (HPC) and enhancing engraftment of long term repopulating cells [17,18]. Interestingly, DEK added to cells is taken up in a bioactive form, moved to the nucleus and re-engages in its bona fide chromatin functions, thus suggesting the existence of a paracrine-loop-like mechanism [19]. Furthermore, DEK works in concert with the transcription factor C/EBP α , whose function can be impaired in AML [20].

DEK also has a long-standing and well-established association with oncogenesis, as it is consistently over-expressed in a number of prevalent and hard-to-treat neoplasms (e.g. retinoblastoma, glioblastoma, melanoma and prostate cancer) [21]. High DEK expression has been shown to directly promote cellular transformation through bypassing major barriers to early oncogenesis and tumor maintenance such as apoptosis and senescence, thus establishing DEK as a bona fide oncogene [22–26]. Furthermore, its expression correlates with metastases and notorious chemoresistance of melanoma and other cancers [22,24,27].

Besides the expression of the DEK-NUP214 fusion gene, two previous studies have indicated that DEK itself is over-expressed in AML [28,29]. In one study, DEK expression profiling was analyzed at diagnosis of 15 primary AML patients with normal and complex karyotypes [28] and quantitative reverse transcription-PCR (qRT-PCR) suggested that DEK was over-expressed independently of karyotype in nine of these cases (60%). Similarly, a qRT-PCR approach showed DEK over-expression in 98% of cases from a cohort of 41 AML patients. Higher levels of DEK were associated with CD34 negative bone marrow samples and independent of the t(6;9) chromosomal translocation [29]. Conversely, DEK expression has been found to be diminished in pediatric AML in comparison to normal bone marrow [26]. In addition, a study of 14 acute promyelocytic leukemia (APL) patients harboring the t(15;17) translocation revealed a non-significant four-fold down-regulation of DEK expression [30]. Overall there are conflicting data regarding the expression status of DEK in AML patients both with or without the t(6;9) translocation.

Although aberrant expression of DEK has been associated with AML, its role in the development of leukemia independent of the t(6;9) translocation remains largely unknown. Previously, DEK expression was reported to be 10-fold lower in mature hematopoietic cells as compared

to immature CD34 positive cells [6]. Since four studies analyzing DEK expression in leukemia were inconclusive the aim of this study was to characterize DEK expression in a large multi-center cohort of AML cases. As an initial reference, DEK expression was profiled during normal hematopoietic differentiation of the myeloid lineage in both human and mouse using the Hemaexplorer database [31]. Analysis of DEK expression in primary AML samples was compared to normal bone marrow using both the Microarray Innovations in Leukemia (MILE) study [32] and acute myeloid leukemia dataset (LAML) from Ley *et al* [33] and mapped back to the normal hematopoietic expression. This was validated and confirmed in independent cohorts of primary AML patient samples at the RNA level by qRT-PCR and at the protein level by immunohistochemistry using a newly assembled AML-specific tissue microarray (TMA). Finally, DEK expression was evaluated in relation to overall survival of AML patients and prognostic relevance using the LAML dataset [33].

Materials & methods

DEK expression profiling in normal hematopoiesis

DEK expression during normal hematopoiesis in both human and murine models was assessed using the publicly available Hemaexplorer database (<http://servers.binf.ku.dk/hemaexplorer>) [31], which enabled DEK gene expression levels to be profiled in hematopoietic cells during different maturation stages based on curated microarray data. The data was analyzed using the Partek Genomics Suite v 6.6 (Partek Inc., Missouri, USA) and GraphPad Prism 5 (GraphPad, California, USA). All data was normalized and batch corrected.

DEK expression profiling in AML

DEK expression levels in AML compared to normal bone marrow (NBM) were determined using the Affymetrix CEL files generated for the MILE study database GSE13204 [32] and the LAML dataset [33], and analyzed using Partek Genomics Suite v 6.6. ANOVA was carried out on microarray results by comparing DEK expression in NBM controls to leukemia in addition to comparison tests between NBM and specific AML subtypes. Overall patient survival associated with DEK expression was analyzed using the alternative microarray dataset LAML generated as part of The Cancer Genome Atlas (TCGA; [33]).

Reverse transcription and quantitative PCR

RNA was extracted and purified from samples of 30 patients with AML (OREC 08/NIR01/9). Synthesis of cDNA was performed using the High-Capacity cDNA reverse transcription (RT) kit according to the manufacturer's protocol (Applied Biosystems, California, USA). RT was performed using the Veriti Thermal Cycler (Applied Biosystems) at the following conditions: 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 min and a 4 °C hold period. All qRT-PCR was executed using the SYBR green mastermix (Roche) on the 7900HT Fast Real-time PCR platform (Applied Biosystems) with standard cycling conditions (95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s). The following primers were used to analyze DEK mRNA expression levels: DEK forward primer ACGGAACAGTCTCTGGAATGG and DEK reverse primer TTTTGGTGCTCTCTTAC.

Immunohistochemistry procedures

In total 122 AML cases (57 female, 65 male with an average age of 60 years) and age-matched bone marrow samples from tumor-free NBM were used to build TMAs, utilizing a semi-automated tissue arrayer (TMArrayer, Pathology Devices, Westminster, MD, USA). The donor tissues were archived bone marrow biopsies and were included in this study using pseudonymized numbers, including tumor entity, gender,

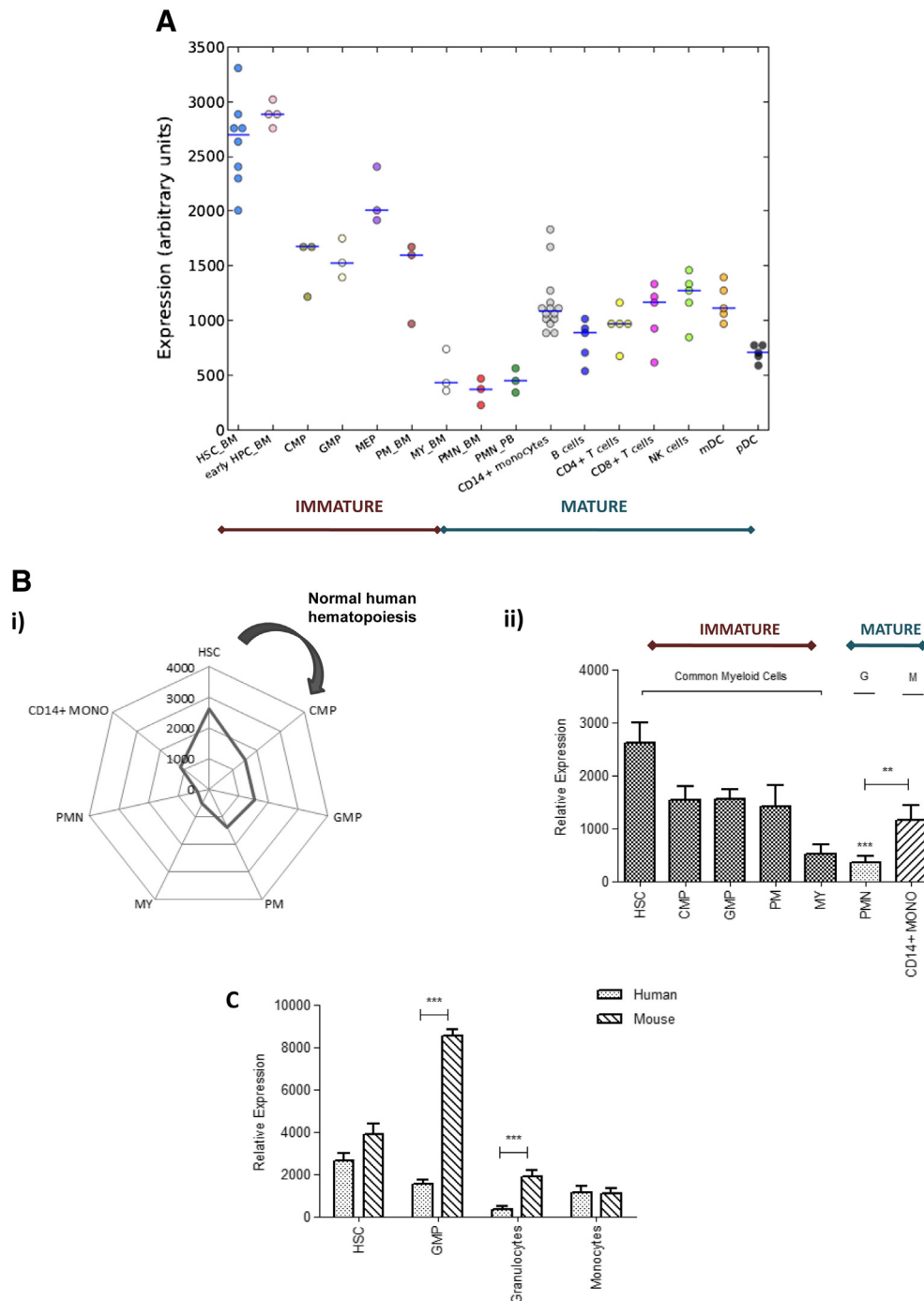


Fig. 1. Differential DEK expression during human hematopoiesis. A. Dot plot representing relative DEK expression during the maturation process of normal human hematopoiesis from immature HSC to mature cell types in myeloid and lymphoid lineages using the Hemaexplorer database. B. (i) Radar plot indicating differential DEK expression throughout myeloid specific normal hematopoietic differentiation. Each radius represents a particular hematopoietic cell stage. (ii) Bar chart highlighting DEK expression during normal human differentiation from the common myeloid cells towards the granulocytic (G) and monocytic (M) lineages. *** $p < 0.001$ and ** $p < 0.01$. C. Comparison of DEK expression in HSC, common progenitors, mature granulocytes and mature monocytes between normal human and murine models of hematopoiesis. All values represent relative mRNA expression levels. Abbreviations as follows: hematopoietic stem cell (HSC), hematopoietic progenitor cell (HPC), common myeloid progenitor (CMP), granulocyte monocyte progenitor (GMP), megakaryocyte erythroid progenitor (MEP), promyelocyte (PM), myelocyte (MY), polymonuclear cells (PMN), natural killer cells (NK) and dendritic cells (DC).

and age with the permission of the ethical review committee of the RTWH Aachen University. Archived paraffin blocks were used and recipient paraffin blocks were heated for 4 h at 40 °C to prevent cracks and missing cores. Subsequently, 3 μ m sections were produced and dried overnight. Immunohistochemical staining was performed by using a heat induced antigen retrieval method in citrate buffer (pH6) (DAKO, Carpinteria, CA; USA), followed by blocking of endogenous peroxidase activity by hydrogen peroxide solution (3%) and blocking of

unspecific protein binding sites (milk powder, 1%). The primary antibody (monoclonal DEK antibody, BD Transduction Laboratories, 610948) with a dilution of 1:400 was incubated on the slides for 1 hour. After washing, the biotinylated secondary antibody was incubated for 30 min followed by the streptavidin–horseradish peroxidase complex (30 min) and chromogene (DAB, 3 min), which were components of the LSAB + Kit (DAKO, Carpinteria, CA, USA). Slides were counterstained with hematoxylin and dehydrated

before the addition of coverslips. The staining was scored by an experienced pathologist (T.B) as an overall staining intensity (staining, numbers of cells) using a semi-quantitative scale, 0–3 in 0.5 increments.

Statistical analysis

Two-way ANOVA or Student *t*-tests were performed on all data using GraphPad Prism 5 software (GraphPad, California, USA). Differences of $p < 0.001$ (***) indicate strong statistical significance, $p < 0.01$ (**) significant, $p < 0.05$ (*) a weak statistical significance and $p > 0.05$ (n.s.) denotes no statistical significance.

Results

DEK expression during normal hematopoiesis

As a crucial prerequisite for the analysis of potentially altered DEK expression in AML samples, we first characterized the expression profile across normal hematopoietic differentiation. A comprehensive *in silico* analysis of human hematopoietic cells revealed that DEK expression was elevated in immature HSCs and diminished markedly in mature myeloid cells present in the peripheral blood and bone marrow (Fig. 1A). DEK expression decreased in a steady stepwise progression in the myeloid lineage with the lowest DEK levels observed in mature cells of the granulocyte lineage, predominantly the polymononuclear cells (PMNs), which exhibited a seven-fold lower expression as compared to immature HSCs ($p < 0.001$) (Fig. 1Bi & ii). Although both granulocytes and monocytes exhibited a reduced level of DEK as compared to HSCs, monocytes were found to express three-fold more DEK compared to normal granulocytes ($p < 0.01$) (Fig. 1Bii).

It is unknown if the DEK expression profile we observed during human hematopoietic differentiation is similar to that of other species, such as mice; a commonly used model. The function of DEK in HSCs has previously been partially elucidated in murine models but the expression profile during murine hematopoietic differentiation has not been characterized. Thus an *in silico* analysis of murine hematopoietic stem cells and progenitors was carried out and compared to that of human hematopoiesis. DEK expression was found to increase from immature long term HSCs (LT-HSCs), reaching a peak at the common progenitor stage namely the granulocyte monocyte progenitor (GMP) before diminishing below its initial expression levels in the mature, terminally differentiated cells (Supplementary Fig. 1A & B). This was in contrast to normal human hematopoiesis, which displayed a decline with no peak in expression at the common progenitor stage. In the myeloid lineages there was a steady incline of DEK expression in common myeloid cells during normal murine hematopoiesis, with a three-fold increase in DEK expression at the GMP cell stage relative to HSCs ($p < 0.001$). However, compared to the LT-HSCs, DEK expression dropped to a three and two-fold lower level in mature granulocytes and monocytes respectively, (Supplementary Fig. 1Bi and ii). Comparison of DEK levels in mature myeloid cells indicated a small difference of 1.5-fold between granulocytes and monocytes, with granulocytes exhibiting higher DEK expression (Supplementary Fig. 1Bii). Analysis of DEK expression at different stages during myeloid differentiation in human and murine cells revealed significant differences at the

GMP and granulocyte stages, while levels in monocytes were similar (Fig. 1C).

DEK expression levels are reduced in AML

To determine if the expression of DEK in AML was aberrant compared to normal hematopoietic differentiation, DEK levels in unfractionated bone marrow derived from 542 AML patients and 74 normal controls were analyzed using the MILE study. A lower, yet not significant, DEK expression across all AML subgroups combined was seen as compared to NBM (Fig. 2A). Since a previous study had already indicated that the APL subgroup of AML exhibited lower DEK expression, the MILE data was further categorized into different AML subtypes, as available, and DEK expression re-analyzed. As observed in the unsorted AML cases, elevated DEK expression was not found in any of the AML subtypes as compared to NBM (Fig. 2Bi). In contrast, all subtypes including 11q23 translocations, normal and other cytogenetics as well as those with balanced recurrent translocations of t(8;21), and t(15;17) displayed significantly reduced DEK expression compared to NBM ($p < 0.005$) with the exception of inv(16) (Fig. 2Bi & Table 1). These findings were further confirmed in a second AML dataset [33], which showed similarly reduced DEK expression levels across all AML subtypes as compared to those in the MILE dataset (Fig. 2Bii). This data was finally compared to AML data from the Hemaexplorer database. DEK was found to exhibit a comparable or reduced level of expression to the common promyelocyte stage of normal myeloid differentiation, which is indicative of immature myeloblasts that accumulate in leukemia (Supplementary Fig. 2). Furthermore, when levels of DEK expression were normalized to that of myeloblasts (equivalent to the closest normal counterpart of myeloid cells), DEK was significantly under-expressed in AML, as indicated by a relative mean value less than 1, which was particularly prominent in the APL sub-type (Fig. 2C).

Expression profiling of primary AML samples confirms that DEK is not over-expressed in AML

This section and Fig. 3 should be in the main text of the Results Section after “DEK expression levels are reduced in AML”. To validate the *in silico* results, we measured DEK expression by qRT-PCR in a separate and independent cohort of defined primary AML samples. Patient characteristics of this primary AML sample cohort are outlined in Supplementary Table 1. DEK expression was found to be similar in 30 AML samples and the 5 NBM, with no significant change in the ΔC_t between NBM and AML observed (Fig. 3A). To establish if DEK expression was independent of varying AML subtypes, samples were further divided into the following subgroups: normal karyotype, promyelocytic leukemia (chromosomal translocation t(15;17)), core binding factor leukemia (chromosomal aberrations t(8;21) and inv(16)), and others, which included 11q23 translocations and complex karyotypes. DEK expression remained similar across all AML subgroups with no significant change in expression between each AML subtype when compared to each other or between individual subtypes and NBM (Fig. 3B).

DEK protein levels are low in AML bone marrow

Although DEK mRNA levels were reduced or remained unchanged it is possible that this does not correlate with protein levels as little is known

Fig. 2. DEK expression levels are under-expressed in AML. A. Box and whisker plot comparing the expression of DEK between normal bone marrow (NBM, $n = 74$) (green) and acute myeloid leukemia (AML, $n = 542$) (red) using the MILE study database. Y-axis represents log2 expression levels. B. (i) Box and whisker plots comparing DEK expression levels of AML subtypes (11q23, complex, Normal, Other, inv(16), t(15;17) and t(8;21)) to NBM using the MILE study database. Y-axis represents log2 expression and p -values are outlined in Table 1. *** $p < 0.001$ comparing AML subtypes to NBM (green). (ii) Box and whisker plots comparing DEK expression levels between AML subtypes (Complex cytogenetics, Normal cytogenetics, Other, inv(16), t(15;17) and t(8;21)) using the LAML study database. Y-axis represents log2 expression. C. Hemaexplorer dot plot depicting DEK expression levels in AML compared to promyelocytes considered as the closest normal counterpart. Grey dashed lines represent DEK expression closest to normal counterpart in normal hematopoiesis with a relative expression value of 1. Samples exhibiting DEK over-expression are indicated by the red arrow and a relative expression greater than 1 while samples displaying reduced levels of DEK are represented by the blue arrow with a relative expression lower than 1.

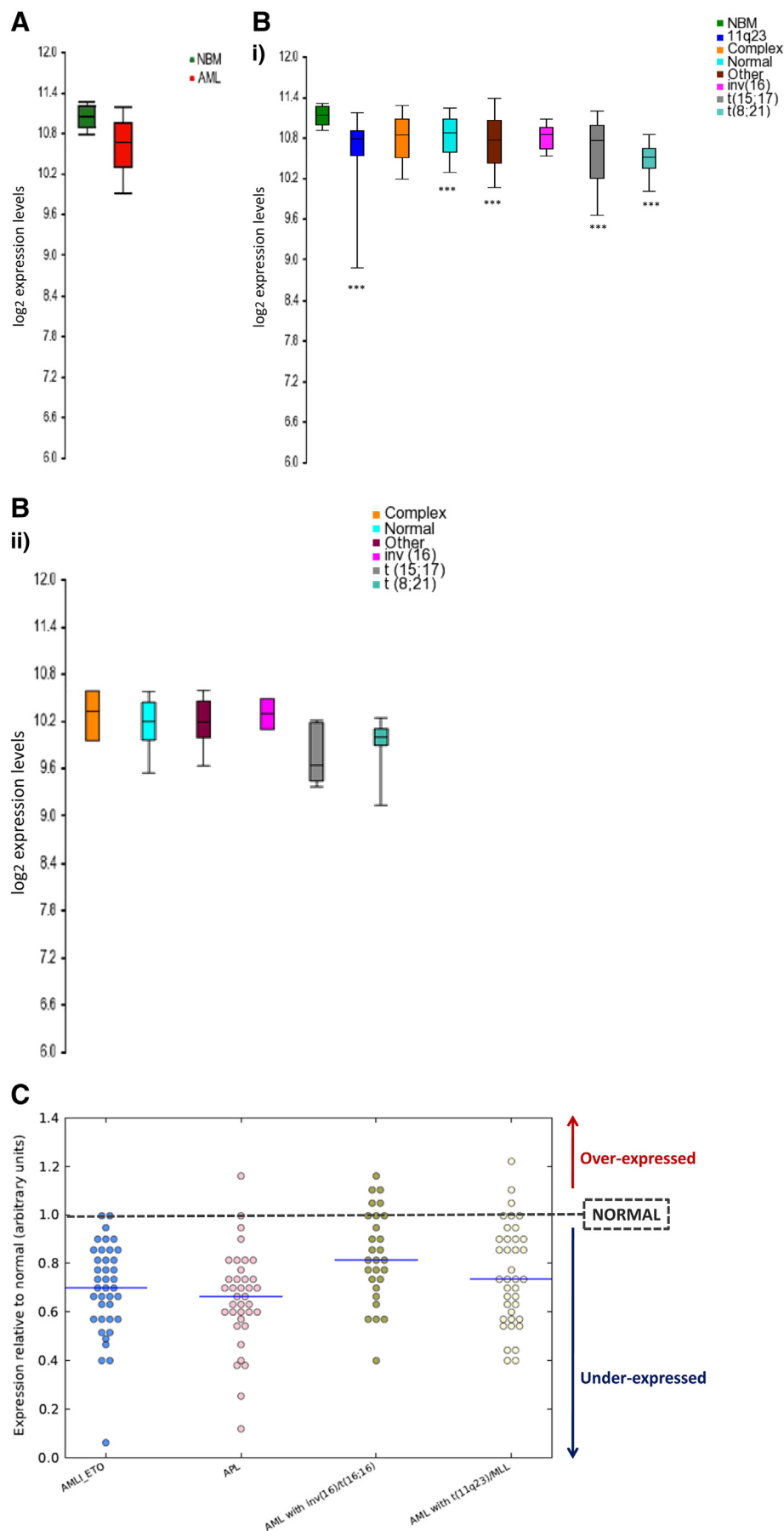


Table 1

Summary of significantly reduced DEK expression in AML.

Comparison of DEK expression levels in AML subtypes (11q23, complex cytogenetics, Normal karyotype, Other, inv(16), t(15;17) and t(8;21)) to NBM using the MILE study database. Data summarized by median expression similar to NBM or median expression lower than that of unfractionated NBM. *p*-values indicated in final column.

	NBM vs subtype	DEK expression change compared to NBM	<i>p</i> -value
Favorable	inv (16)	Similar expression to NBM	0.099
	t(15;17)	Median expression lower than NBM	6.042 e – 6
	t(8;21)	Median expression lower than NBM	1.231 e – 5
Intermediate	Normal karyotype	Median expression lower than NBM	0.004
	Other	Median expression lower than NBM	0.001
	11q23	Median expression lower than NBM	0.001
Adverse	Complex karyotype	Similar expression to NBM	0.023

about the post-transcriptional cues that regulate DEK mRNA. Since we were particularly interested to validate our findings at the protein level a novel custom-built TMA was assembled. The TMA utilized bone marrow biopsies from 122 AML patients and 20 age-matched bone marrow samples from tumor-free normal bone marrow, which were allocated from the Biobank at the University Clinic of the RWTH Aachen University. All samples were spotted in triplicate, including appropriate positive and negative controls, to produce five TMA slides in total. The slides were subjected to immunohistochemistry using a monoclonal DEK-specific antibody (Fig. 4). We observed a strong DEK-specific nuclear signal in a colon biopsy, which served as a positive control for the specificity of the antibody (Fig. 4A-1). In contrast, the DEK antibody produced a rather weak, diffusely cytoplasmic staining, which was seen mainly in myeloid progenitor cells, in 90% of normal bone marrow biopsies from tumor-free patients (Fig. 4A-2 and B). The majority of AML samples presented with a similar outcome of none or weak nuclear staining with mostly none (score 0) (Fig. 4A-3 and B) or weak (score 0.5) (Fig. 4A-4 and B) DEK staining as determined by pathology assessment. Around 10% of the AML biopsies showed a moderate staining (score 1, Fig. 4A-5 and B), and only less than 5% of all AML samples exhibited a strong nuclear staining (score 2; Fig. 4A-6 and B). Thus, DEK expression at the protein level was in agreement with the data obtained at the mRNA level in the other AML cohorts.

Reduced DEK expression does not influence overall survival of AML patients

Since overall reduced and parallel expression of DEK both at the RNA and protein level was found in AML, it is possible that DEK may have

prognostic relevance for the long term survival of AML patients. Using leukemia microarray datasets 164 patients with DEK expression were stratified into four equal quartiles of 40 patients with Quartile 1 exhibiting the lowest DEK expression and Quartile 4 representing the highest DEK expression (Table 2). Overall survival of patients in each quartile was independent of DEK expression (Supplementary Fig. 3Ai). Additionally, Kaplan–Meier curves plotting DEK expression above and below the median indicated that the overall survival of patients was identical regardless of low or high DEK levels (Supplementary Fig. 3Bi). The Kaplan–Meier curves show that Quartiles 1–3 combined exhibited an increased, but insignificant survival benefit compared to those patients in Quartile 4 with the highest DEK expression levels (Fig. 5A). Based upon the long term survival of AML patients it is possible to divide AML into 3 risk groups, favorable, intermediate and adverse. Although all quartile groups contained patients from each risk group, the favorable risk group patients were more prevalent in Quartiles 1 and 2 while the remaining quartiles are mainly composed of the intermediate risk group (Table 2). Removing the favorable risk group from the analysis which includes patients harboring the recurrent balanced translocations including t(15;17), t(8;21) and inv(16), and replotting the Kaplan–Meier curves resulted in identical long term survival between high (Quartile 4) and low levels (combined Quartiles 1–3) of DEK expression (Fig. 5B). Similarly, no difference in overall survival was observed with removing the favorable risk group from the individual quartiles or when comparing DEK expression above and below the median levels (Supplementary Fig. 3 A&Bii respectively). The favorable group, which can be treated with all-*trans* retinoic acid (ATRA), contains the acute promyelocytic leukemia patients with

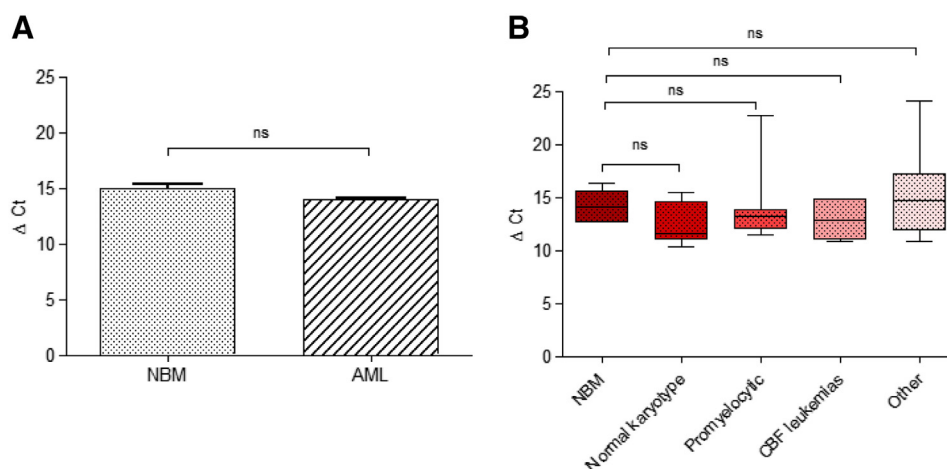


Fig. 3. DEK expression profiling in primary AML samples. A. qRT-PCR analysis of DEK expression in 30 primary AML patient samples compared to 5 unfractionated NBM controls B. qRT-PCR analysis comparing DEK mRNA expression levels in unfractionated NBM to primary AML samples subdivided into different AML subtypes including normal karyotype $n = 7$, promyelocytic leukemia (t(15;17)) $n = 12$, core binding factor leukemias (t(8;21) and inv(16)) $n = 5$ and other (11q23 and complex) $n = 6$. Y-axis of both plots represents ΔCt whereby high ΔCt indicates low expression levels and low ΔCt translates to high expression levels. 18S was used as an endogenous control.

translocation t(15;17) and core binding factor aberrations including translocation t(8;21) and inv(16). Thus it appears that DEK expression does not influence patient survival independent of the favorable risk group of AML patients.

Discussion

In this report, DEK expression was comprehensively analyzed during normal human hematopoietic differentiation for the first time. DEK expression was found to be the highest in human hematopoietic stem cells but decreased during myeloid differentiation up to 7-fold in granulocytes (Fig. 1). This broadly agreed with the detection of a 10-fold lower expression of DEK in mature cells from peripheral blood compared to normal CD34+ cells as revealed in a previous study [6]. However, not all terminally differentiated cells from different hematopoietic lineages exhibited similar expression of DEK, as higher DEK levels were observed in lymphoid cells as compared to mature myeloid cells. Within the myeloid lineage, monocytes had a 3-fold higher DEK expression than granulocytes (Fig. 1). Since DEK could be important in regulating granulocytic differentiation it may be expected that its expression could subsequently promote terminal differentiation in AML. In contrast, mice exhibited a markedly different expression pattern compared to that of humans (Fig. 1C & Supplementary Fig. 1). Most significantly, murine cells expressed elevated levels of Dek in GMPs and mature granulocytes as compared to the human myeloid cell equivalent ($p < 0.001$). However, DEK expression levels in monocytes were similar (Fig. 1C). Overall, distinct DEK expression patterns were observed during the progression of normal hematopoiesis, with DEK levels substantially reduced in mature

cells compared to HSCs. Thus it appears that DEK levels during murine and human hematopoiesis highlight potential differences which may reflect cell type specific functions of DEK. However, the precise function of DEK in myeloid proliferation/differentiation remains unknown and requires further elucidation.

Since DEK is generally found up-regulated in multiple human malignancies and is associated with the AML subgroup harboring the t(6;9) translocation it is possible that AML may also exhibit up-regulated DEK. However, four previous studies analyzing DEK expression in AML have given discordant results with over-expression in two studies and either no significant change or decreased expression in the others. Consequently this study aimed to clarify the expression status of DEK in AML. Analysis of DEK expression in three datasets of AML patients indicated that DEK was not over-expressed and may actually be under-expressed in the majority of cases. Furthermore, dividing the AML patients into different subtypes detected no significant change or decreased DEK expression (Fig. 2). In agreement with our findings, a previous study of 14 APL cases, which possess the t(15;17) translocation and have a favorable prognosis, showed that there was no significant change in DEK expression. Analysis of over 500 pediatric AML samples from the Oncomine dataset [26], combined with over 600 adult samples in the MILE and LAML studies plus collated microarrays from the Hemaexplorer dataset, totaling more than 1000 cases of AML, supported an association of reduced DEK expression in AML. Although the median DEK expression was normal or reduced there was a range of expression levels with a few highly expressed outliers (Fig. 2 & Supplementary Fig. 2). Importantly, this observed pattern of reduced DEK expression was also seen at the protein level using a distinctively different AML

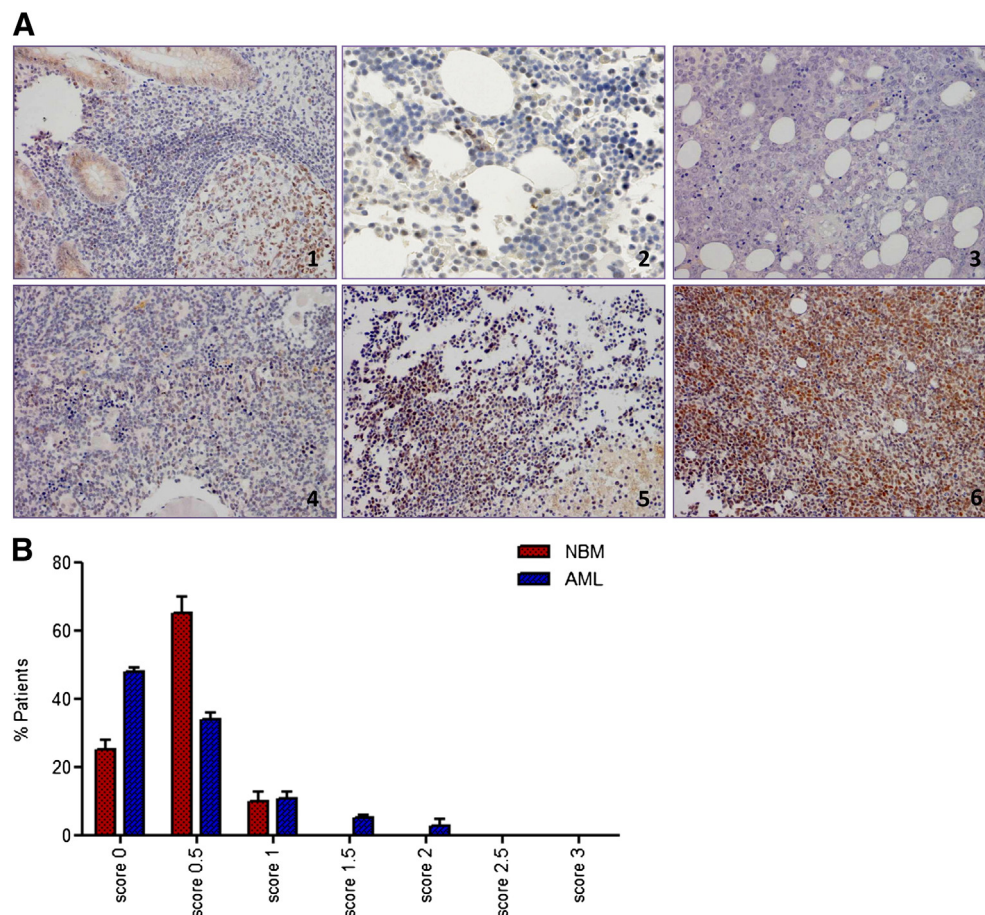


Fig. 4. DEK protein expression is also diminished in AML. A. Immunohistochemistry analysis of a custom-built AML TMA. Shown are representative micrographs. 1: colon biopsy with strongly DEK-specific nuclear staining; 2: normal bone marrow from tumor free patients with very weak and diffuse DEK staining; 3–6: AML samples with no DEK staining (3: score 0); weak DEK staining (4: score 0.5); moderate DEK staining (5: score 1) and strong DEK staining (6: score 2). Magnification 400 \times . B. Bar chart depicting percentage of NBM tumour-free and AML patients for each category of DEK stain distribution.

Table 2

Distribution of AML sub-types between quartiles of patients stratified on the basis of DEK expression.

A total of 164 AML patients with DEK expression were stratified into four equal quartiles of 40 patients with increasing DEK levels from Quartile 1 to Quartile 4. Quartile 1 = very low DEK levels, Quartile 2 = low DEK levels, Quartile 3 = moderate DEK levels and Quartile 4 = high DEK levels. Each quartile was represented by patients in all AML subtypes (t(8;21), t(15;17), inv(16), normal karyotype, other and complex cytogenetics) and all AML risk groups (favorable, intermediate and adverse).

	AML subgroup	Quartile 1 Very low DEK	Quartile 2 Low DEK	Quartile 3 Moderate DEK	Quartile 4 High DEK
Favorable	t(8; 21)	1	5	1	1
	t(15; 17)	3	2	1	0
	inv(16)	0	3	3	2
Intermediate	Normal	29	24	22	24
	Other	4	4	6	4
Adverse	Complex	3	2	7	9

cohort that was analyzed by immunohistochemistry on newly created AML TMAs. Also, low levels of DEK expression were observed in most AML blasts, with only a few high DEK expression outliers, fully in agreement with the RNA expression data from the MILE, LAML and

Hemaexplorer. Reduced DEK expression does not appear to be associated solely with pediatric leukemia as previously suggested, supported by the inclusion of exclusively adult cases investigated throughout this study in all datasets and primary AML patient samples. Patients included represent a comprehensive range of AML subtypes and low DEK expression was found to be associated with all AML subtypes, which was verified in an independent cohort of primary samples (Fig. 2).

Low DEK expression may be of prognostic relevance for the long term survival of AML patients but stratifying patients on the basis of DEK expression levels indicated that there was no influence on patient survival either in the presence or absence of the favorable risk group of AML patients (Fig. 5 & Supplementary Fig. 3). The favorable group contains patients with APL, who have a good prognosis as ATRA treatment alleviates the block in differentiation. It is unknown whether DEK contributes to improved survival in the favorable risk group although in the study of APL patients Savli *et al.* [30], similarly to our study, found that DEK expression was reduced by a factor of 4 but this was not statistically significant. However, there is insufficient evidence to establish if DEK levels may have an effect on overall survival of the favorable risk group patients, especially those classified as promyelocytic. Based on the gene expression levels of DEK expression it can be concluded that DEK does not correlate with the survival of AML patients.

In this study we investigated the expression of the DEK oncogene in three independent AML datasets and, contrary to current perception, established that DEK is under-expressed compared to the equivalent normal myeloid cell. However, DEK did not influence overall survival of AML patients. DEK levels in normal hematopoietic differentiation of human and mouse revealed that DEK levels were associated with HPC and specific cell stages, hence suggesting distinct functions during myeloid differentiation for DEK. Although the precise function of DEK in myeloid proliferation and differentiation remains unknown, DEK may be playing an important role in hematopoiesis. However, it remains to be established whether DEK is important for leukemagenesis, except when involved in the t(6;9) translocation.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bcmd.2014.07.009>.

Acknowledgments

This study was supported and funded by Leukaemia & Lymphoma N1R2561CNR (GEL, KIM, MJP), the START-Program of the Faculty of Medicine, RWTH Aachen University (to F.K.) and the German Research Foundation (DFG; KA 2799/1 to F.K.).

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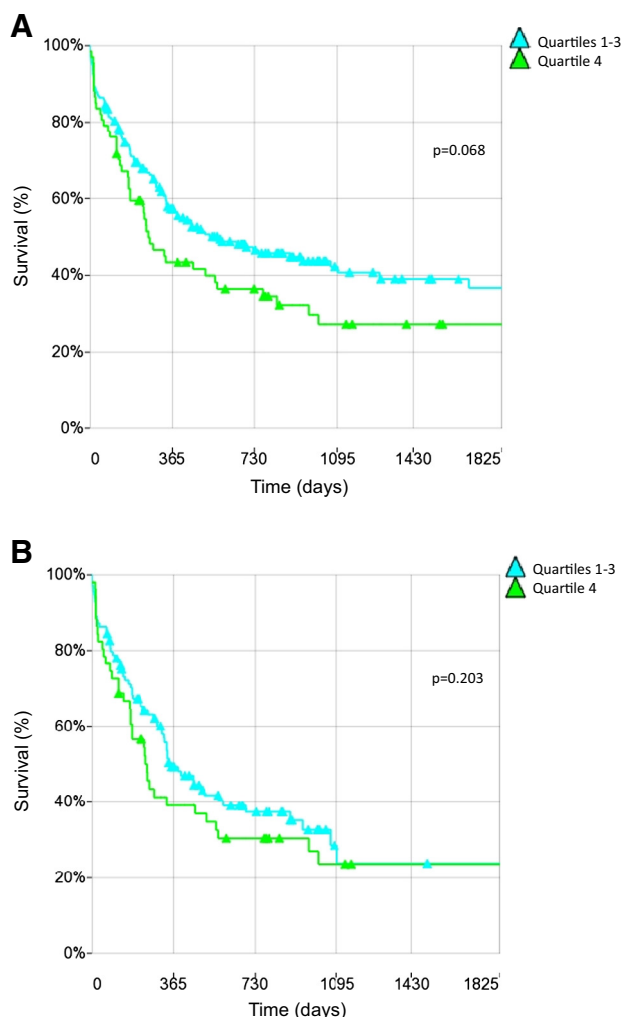


Fig. 5. DEK expression is not associated with overall survival of AML patients. A. Kaplan-Meier overall patient survival curves plotted for low DEK levels (Quartiles 1–3 combined) compared to high DEK expression (Quartile 4) survival. B. Removing the favorable risk group from the analysis and re-plotting the Kaplan–Meier curves comparing overall survival between low DEK levels (Quartiles 1–3 combined) and high levels of DEK (Quartile 4). *p*-values outlined on each plot.

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